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A dynamic scaffolding mechanism for rhodopsin and transducin interaction in vertebrate vision

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Short title:

dynamic scaffolding in rhodopsin-transducin interaction

Synopsis

The early steps in vertebrate vision require fast interactions between rhodopsin (Rho) and transducin (G_t), which are classically described by a collisional coupling mechanism driven by the free diffusion of monomeric proteins on the disc membranes of rod and cone cells. Recent findings, however, point to a very low mobility for Rho and support a substantially different supramolecular organization. Moreover, Rho- G_t interactions seem to possibly occur even prior to light stimuli, which is also difficult to reconcile with the classical scenario.

We investigated the kinetics of interaction between native Rho and G_t in different conditions by surface plasmon resonance and analysed the results in the general physiological context by employing a holistic systems modelling approach. Our data point to a mechanism, which is intermediate between pure collisional coupling and physical scaffolding. Such a “dynamic scaffolding”, in which prevalently dimeric Rho and G_t interact already in the dark by forming transient complexes (~25% of G_t precoupled to Rho) does not slow down the phototransduction cascade but is compatible with the observed photoresponses on a broad scale of light stimuli. We conclude that Rho molecules and Rho- G_t complexes can both absorb photons and trigger the visual cascade.

Keywords:

kinetics, mathematical modelling, phototransduction, precoupling, systems biology

Introduction

Phototransduction in vertebrates represents a paradigm for G protein-coupled receptor (GPCR) mediated signalling pathways. The cascade is initiated by the absorption of even single photons by the membrane receptor rhodopsin (Rho) and the subsequent catalytic activation of the cognate G protein transducin (G_t) [1].

The molecular events underlying the rapid activation/deactivation of the cascade in response to the broad variety of light stimuli require fast interactions between all the components. Traditionally, such rapid interactions have been explained by a collisional coupling mechanism driven by the free diffusion of monomeric proteins on the disc membranes of rod and cone cells supported by early observations of Rho lateral diffusion on the disc surface [2, 3].

Recent results in high-resolution structural biology, however, appear substantially incompatible with the traditional mechanistic model based on free diffusion. In particular, some lines of evidence seem to be at odds with the classical scenario. First, atomic force (AFM) and electron microscopy indicate that Rho in native mouse discs is organized in highly dense arrays of paracrystalline rafts [4, 5]. Second, recent experiments on Rho diffusion performed by high-speed dichroic microspectrophotometry in native amphibian and gecko rods [6] point to a heterogeneous mobility of Rho in disc membranes. The actual diffusion coefficient of Rho eventually results to be three-to-fivefold lower compared to the classical value, consistent with the existence of a substantial immobile fraction of Rho, whose size can virtually range from 0 to 100% of the disc area, probably reflecting massive oligomeric states of the receptor [6]. Finally, very recent AFM determinations [7] are consistent with Rho being loosely packed in the central zone of the disc membrane, offering again a picture that significantly differs from the classical one.

On the other hand, early studies [8], recent plasmon resonance technology-based findings [9, 10], computational structural analysis [11] and molecular dynamics simulations [12] strongly suggest that Rho and G_t may interact also in the dark, thus further complicating the scenario and possibly pointing to some other mechanism of Rho- G_t interaction. Precoupling between the G-protein and the receptor prior to agonist stimulation was predicted as a possible state in theoretical kinetic analysis of GPCRs [13] and it was experimentally demonstrated for systems like α_2A adrenergic receptors, muscarinic M4 receptors [14] and β_2 -adrenoceptor [15] by *in vivo* studies. Interestingly, phototransduction in *Drosophila*, one of the fastest known signalling systems, was shown to require a scaffolding protein (INAD) that dynamically preassembles parts of the molecular machinery, hence ensuring fast and coordinated visual signaling [16]. Nevertheless, it is arguable that specific features of the phototransduction cascade in vertebrates - the low concentration of G_t compared to Rho and the yet very fast interactions required - make it hardly compatible with a purely precoupled Rho- G_t state. Such a mechanism, if existing, should be a dynamic phenomenon. To properly address the physiological relevance of such a dynamic precoupled Rho- G_t state in the dark it is fundamental to obtain kinetic rather than equilibrium information, but this information was missing until now.

In this work, we show that it is possible to reconcile recent findings on Rho diffusion and assembly with the established activation kinetics of phototransduction by complementing robust biophysical investigation with systems-level analysis of the

cascade dynamics. A surface plasmon resonance (SPR)-based methodology developed previously in our laboratory [17, 18] to functionally immobilize native bovine Rho on a biosensor chip was used to perform interaction kinetic analyses focusing on different states of the receptor. We especially focused on the currently missing kinetic characterization of Rho-G_t dark binding. In order to investigate the extent to which our findings are compatible with the overall physiological process of phototransduction, the experimental information was integrated into a comprehensive quantitative model of phototransduction in rod cells that explicitly includes most of the molecular components of the cascade [19]. The dynamic pre-assembly of Rho and G_t was found to be fully compatible with the experimentally observed photoresponses over a broad range of light stimuli and does not slow-down the photoresponse kinetics. We conclude that an isolated Rho molecule or a Rho-G_t complex involving 25% of the available transducin in the dark can both absorb a photon and normally trigger the cascade.

Experimental

Preparation of Rho and G_t

Bovine rod outer segments were prepared from fresh bovine retinæ and stored at -80 °C as described previously [20]. Rho was extracted from hypotonically stripped disc membranes under dim red light as described [17]. G_t was obtained as described [21] and the buffer exchanged to SPR running buffer by PD10 chromatographic columns (Amersham Biosciences). Protein concentration was determined by Bradford assay [22] and the purity was checked by standard SDS-PAGE. The presence of GDP as well as the absence of GTP was confirmed by HPLC. Freshly prepared aliquots of G_t were frozen and stored at -80 °C until use.

Surface plasmon resonance spectroscopy

Rho-G_t binding SPR experiments were performed with a Biacore 2000 apparatus (GE Healthcare) using CM5 sensor chips, on which Rho was immobilized via ConA and 1D4 monoclonal antibody as described [17, 18]. The instrument was previously modified by addition of a Diode Pumped Nd:Yag solid-state laser emitting at $\lambda = 532$ nm connected to the sensor chip surface through an optical fibre [18]. ConA (Serva, Heidelberg, Germany) and anti-rhodopsin 1D4 monoclonal antibody (Chemicon Europe Ltd, UK) were immobilized at similar levels as Rho was obtained by solubilisation in 25 mM CHAPS at 4 °C for 30 min (gentle shaking) and centrifugation (17,000 rpm) for 15 min at 4 °C to separate it from insoluble material [17] (all these and the following steps were performed in the dark). It was then immobilised on the sensor chip after 5-fold dilution in MES buffer, pH 6.0, 1mM CaCl₂, 1 mM MnCl₂, to a final concentration of approximately 0.4 mg/mL and injected at a flow rate of 2 μ L min⁻¹ on the flow cell for 25 min in the dark. In order to remove unbound Rho and detergent in excess, the system was washed 5 times with running buffer (50 mM MOPS, pH 7.5, 50 mM NaCl, 3 mM MgSO₄, 10 μ M CaCl₂, 10 μ M MnCl₂) and then the signal was detected for other 25-40 min, until a stable baseline was reached.

In each Rho-G_t binding experiment, one flow cell on the sensor chip was used as a blank. This surface contained either ConA or 1D4 at similar levels, but was not coated with Rho. The signal from the blank was subtracted upon each parallel injection of G_t in binding experiments. Kinetics was detected following 60 s injection

of G_t at $10 \mu\text{l min}^{-1}$ (association) and then running buffer only was injected for a time varying from 60 s to 20 min (dissociation). Experiments were repeated several times at different G_t concentrations and Rho immobilisation levels (Table 1). After a series of illumination experiments the pigment was regenerated by flowing $10 \mu\text{M}$ 9-cis retinal for 3.5 h ($5 \mu\text{l min}^{-1}$) followed by repetition of several washes and a ~ 30 min stabilisation of the baseline.

To study the direct interaction of G_t with a lipid bilayer we proceeded as follows. Liposomes were prepared as described [23]. In brief, a mixture of 4 mg lipids with ROS membrane composition (40% v/v phosphatidylethanolamine (PE), 40 % v/v phosphatidylcholine (PC), 15 % w/w phosphatidylserine (PS) and 5% cholesterol) was dried by vacuum in a SpeedVac concentrator. The sample was resuspended in 2mL of degassed buffer 20 mM HEPES, pH 7.5, 150 mM KCl, 3mM EGTA and sonified for 2 X 15 min. Liposomes were produced using the extrusion technique. The suspension was soaked for 15-20 min and extruded through polycarbonate filter with a pore diameter of 100 nm. A sensor chip L1 (GE healthcare) was used to immobilize the liposomes by injecting 150 μL with a flow rate of 5 $\mu\text{L}/\text{min}$ and the stability of the formed bilayer was checked for approximately 1 h before the injections of G_t .

Investigation of the oligomeric state of Rho immobilized on the sensor chip

Experiments aimed at assessing the oligomeric state of Rho immobilized on the sensor chip via ConA or 1D4 were performed after direct immobilization of Rho on the sensor chip via amine coupling (5-fold dilution in Na-acetate buffer 10mM pH 4.3; running buffer: HEPES 10 mM, pH 7.5, 150 mM NaCl, 3mM EDTA, 0.005 % v/v tween 20). Rho was covalently bound to the dextran matrix via free amines located either at its N-terminus or at the side chain of one of the ten Lys residues. These latter are situated mostly at the intracellular region, but not in the transmembrane domain, hence allowing for effective interactions with other Rho molecules through the transmembrane moiety. Reactive carboxyl groups in excess on the dextran matrix were blocked with ethanolamine 1M, pH 8.5. One flow cell was similarly activated and deactivated, without any immobilization of Rho, and used as a blank. In order to study Rho-Rho interactions, the initial dilution step after detergent solubilisation was performed in MOPS running buffer (see above), in order to minimize bulk effects upon injection of Rho. Three minutes Rho injections were performed at different concentrations in the range 1.5-7.6 μM after specific dilutions. The concentration of injected Rho was determined by comparing the absorption at 500 nm in the dark and after complete bleaching, using a molar extinction coefficient equal to $40,600 \text{ M}^{-1}\text{cm}^{-1}$ [24]. The Rho-Rho binding experiments were performed in the dark after removing non-covalently bound Rho from the surface of the sensor chip via a short pulse of 0.25% SDS (5 μl , 30 s). Binding experiments were repeated with different immobilization levels and concentrations of Rho in the flowing phase and the results averaged. In each binding experiment a significant amount of Rho remained bound to the chip, indicative of the formation of a very stable Rho-Rho complex in the tested conditions.

Determination of the delipidation profile of immobilized Rho

By keeping track of the volume of running buffer flowing over the flow cell in which Rho was immobilised during the five washing steps (approximately 342 μL , RINSE protocol in the Biacore protocols, repeated 5 times) and adding the subsequent volume flowed during the stabilization procedure (40 min with high flow of 20 $\mu\text{L}/\text{min}$) the Rho-containing flow cell volume ($\sim 0.02 \mu\text{L}$) was overall flow-dialysed against a 57,000-fold excess volume of running buffer. The delipidation profile was assessed by collecting ten 10 μL -fractions of sample dissociating from the flow cell immediately after the immobilization of Rho via ConA, which occurred after washing the recovery cup with running buffer. Each fraction represents the result of a consecutive 500-fold flow cell volume (FCV) dilution and allows determining the course of delipidation of Rho bound to the sensor chip surface. This latter can be assessed by monitoring the SPR signal in the same time frame in which the fractions are collected. Overall, the 10 fractions collected represent a 5,000-fold flow dialysis of the flow cell volume. Each fraction was analyzed separately to determine the lipid content by Stewart assay [25]. The lipids were determined also in the sample initially used for Rho immobilization and the concentration of lipids in each fraction was related to that initial value (100% of initial lipids) to assess the percent of initial lipid concentration lost in each fraction with the progress of the flow dialysis. The same applied to the amount of Rho lost with each fraction, hence referred to the initial level of Rho immobilized (100% of initial Rho). The delipidation and Rho loss profiles could then be both followed quantitatively at each 500-fold FCV washing step. The experiments were repeated twice.

Kinetic data analysis

Kinetic data were analysed by starting from the dissociation phase as follows:

- a) *dark Rho-G_t binding*: the dissociation process was successfully described as a first order exponential, yielding directly k_{dark}^{off} whose value was fixed in a standard pseudo-first order reaction to yield the fitting of the association SPR response according to:

$$RU(t) = R_0 + \frac{k_{dark}^{on} [G_t] R_{max}}{k_{dark}^{on} [G_t] + k_{dark}^{off}} \left(1 - \exp\left(-\left(k_{dark}^{on} [G_t] + k_{dark}^{off}\right)t\right) \right) \quad (1)$$

in which k_{dark}^{on} is the association rate constant, R_0 is the baseline value and R_{max} indicates the maximum response expected if all the injected G_t bound Rho (saturation);

- b) *Rho*-G_t binding*: in order to account for the concomitant processes of G_t binding to Rho and Rho*, the dissociation phase was fitted as the sum of two exponential decays:

$$RU(t) = R_1 \exp(-k_{dark}^{off} t) + R_2 \exp(-k_{light}^{off} t) + R_3 \quad (2)$$

in which k_{dark}^{off} was fixed to the average value determined in dark binding experiments and k_{light}^{off} was determined by the fitting procedure (other

parameters are for the baseline and the number of Rho* following the stimulus). Accordingly, the association phase was fitted by a sum of two pseudo-first order terms:

$$RU(t) = \frac{k_{dark}^{on} [G_t] R_{dark}^{max}}{k_{dark}^{on} [G_t] + k_{dark}^{off}} \left(1 - \exp\left(-\left(k_{dark}^{on} [G_t] + k_{dark}^{off}\right)t\right) \right) + \frac{k_{light}^{on} [G_t] R_{light}^{max}}{k_{light}^{on} [G_t] + k_{light}^{off}} \left(1 - \exp\left(-\left(k_{light}^{on} [G_t] + k_{light}^{off}\right)t\right) \right) + R_0 \quad (3)$$

in which k_{dark}^{on} , k_{dark}^{off} and k_{light}^{off} were fixed according to the values determined above in independent fittings.

- c) *Ops*-G_t binding*: the dissociation phase and the association phase were successfully fitted according to a single exponential and a pseudo first order exponential, respectively, yielding directly k_{Ops}^{off} and k_{Ops}^{on} , as done for the dark Rho case.

The kinetic constants obtained in each experiment were averaged and the mean and its standard error were reported (Table 1). The equilibrium dissociation constant K_D for each rhodopsin variant was computed as the ratio between the k^{off} and the k^{on} values (Table 1). In order to transform the volume-related constants into more convenient surface-density (2D) units of physiological relevance as referred to the situation on rod discs surface, we used the relation elucidated in ref. [26]:

$$K_D^{2D} = \frac{[R]_{disk}^{2D}}{[R]_{chip}^{3D}} \times K_D \quad (4)$$

in which K_D is the volumetric dissociation constant obtained as described, $[R]_{disk}^{2D}$ is the average concentration of Rho on the disc surface ($48,000 \mu\text{m}^{-2}$, Ref. [4]) while $[R]_{chip}^{3D}$ is the volumetric concentration of the Rho immobilized on the sensor chip (on average $12.4 \mu\text{M}$ in all the experiments considering a $0.02 \mu\text{L}$ volume for the flow cell).

Numerical Simulations. The mathematical model of rod phototransduction including 91 reactions, 71 molecular components and 63 parameters previously developed [19] (BioModels ID: BIOMD0000000326) and validated over a broad range of experimental data on normal and genetically manipulated rods was modified by inserting a reversible precoupling reaction with the kinetic constraints resulting from SPR experiments, that is:



in which the kinetic rate constants were constrained to be $k_{dark}^{on} = 1.6 \times k_{light}^{on}$ and $k_{dark}^{off} = 315 \times k_{light}^{off}$, according to SPR data (Table 1).

Similar to previous work [19], the stimulus was set to define the velocity of the activation reactions in the cascade, and was defined as a flash of specific duration and intensity:

$$stimulus = \frac{flashMag}{flashDur} \quad (6)$$

where flashMag is the potential number of photoisomerizations led by the flash, proportional to its intensity, and flashDur (24 ms) is the duration of each flash. Based on such definition, the number of photoactivations of either Rho or Rho-G_t preformed complexes will be proportional to the stimulus and the relative abundance of the pigment form that defines the probability of the process. In the case of the preformed complexes the following rate expression stands:

$$\frac{dRho \cdot G_t^*}{dt} = stimulus \times \frac{Rho \cdot G_t}{Rho_{TOT}} \quad (7)$$

where the number of molecules rather than their molar concentration are considered, and Rho_{TOT} represents the pigment overall available in the cell, independent of its binding status.

Parameter estimation for k_{light}^{off} , k_{light}^{on} and k_{RKO} (the rate constant of unphosphorylated Rho to rhodopsin kinase binding) was necessary and sufficient to reproduce the benchmark experiments described in Ref. [19]. The estimated parameters were within a factor of two compared with the prior values reported in Ref. [19], and none of the remaining 60 parameters needed to be newly estimated, indicative of robustness of the proposed network.

All the numeric simulations were performed in Matlab, within the SBTOOLBOX2 framework [27] (<http://www.sbtoolbox2.org/main.php>) as describe earlier [19].

Results and Discussion

G_t interacts with every Rho state with specific kinetics.

Native bovine Rho and G_t were extracted from dark-adapted retinae.. Two different coupling strategies, which have been deeply investigated previously [17] were used to immobilize detergent-solubilised Rho on the surface of a biosensor chip at sufficient concentration (average density of 0.2 pmol mm⁻², corresponding to approximately 12 μM concentration), namely: a) immobilization via high affinity binding of the carbohydrate moiety of Rho to the lectin Concanavalin A (ConA) [18]; b) immobilization via 1D4 monoclonal antibody, which recognizes the last 8 amino acids of the C-terminal tail of Rho [17]. Our recent results employing the same immobilization strategies pointed out that no significant difference in the kinetics and in the affinity of Rho*-G_t interaction is detected between the two coupling techniques [17]. This unexpected result suggests that Rho is dimeric on the surface of the sensor chip, and while one monomer is involved in the coupling with the chip through either of its terminals, the other one is free to interact with G_t with an ideal stoichiometry. In order to test such hypothesis we performed Rho-Rho binding experiments after immobilizing Rho on the sensor chip via direct amine coupling. The coupling was heterogeneous, as any amine group, at the N-terminus or at Lys side

chains, might couple to the dextran matrix. However, the spatial distribution of such groups is mostly at the intracellular domain (apart from Lys16, which is in the intradiscal region), thus keeping the transmembrane region free for interaction with another Rho molecule. After the direct immobilization of Rho, a short pulse of detergent (0.25% SDS, 30 s) was injected in order to dissociate potential oligomeric forms of Rho formed on the chip, resulting in a significant reduction of the SPR signal (>80%), indicative of a substantial dissociation process. After checking for baseline stability, injections of Rho in the range 1-8 μM were performed, resulting in a slow but stable binding, in which a significant amount of injected Rho remained bound to Rho previously immobilized on the chip (Figure 1). Short pulses (30 s) of regeneration buffer (MOPS buffer containing 100mM methyl- α -D-mannopyranoside and 30mM octyl- β -D-glucopyranoside) following the binding experiments allowed a significant dissociation of high-affinity Rho-Rho complexes. Taking into account the bulk effect due to the presence of detergent in the injected sample, the data were effectively fitted with a simple 1:1 binding model, leading to a submicromolar affinity (on average 17 nM for 4 repeated experiments). Therefore, we conclude that most, if not all, of the Rho immobilized via ConA or 1D4 and used in G_t binding experiment was dimeric, as hypothesized.

For the Rho- G_t interaction experiments, the immobilization and binding steps were performed in the dark except for those concerning the illumination of the sensor chip by pulses of green light ($\lambda = 532 \text{ nm}$) delivered via an optical fibre connected with a laser source as described [18]. In every case, bleached pigments could be regenerated with 9-cis retinal after any cycle of repeated illumination yielding 80-90% of the former response. The purity of heterotrimeric G_t samples used for binding experiments was confirmed by SDS-PAGE (Figure 2a)

Figure 2b shows an overlay of representative sensorgrams obtained after 60 s injection of 0.33 μM G_t over 0.22 pmol mm^{-2} Rho immobilised on the sensor chip. Further flowing only running buffer for 60 s monitored the dissociation of the protein-protein complex. The observed kinetics was found to strongly depend on the receptor state. Very fast associations and dissociations were observed when the binding experiments were performed in the dark (dotted black line in Figure 2b). The measured rate constants are reported in Table 1. The high association rate ($k_{\text{dark}}^{\text{on}} = 4.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) is paralleled by an extremely fast dissociation ($k_{\text{dark}}^{\text{off}} = 0.148 \text{ s}^{-1}$), leading to a relatively high 0.36 μM affinity, which is in line with the values reported in earlier equilibrium studies (64 nM – 10 μM)[9, 10, 28]. When the same Rho sample was illuminated for 4 s, sensorgrams showed a significantly higher response amplitude (Figures 1b and 1c, black solid line) with a slightly slower association and a significantly slower dissociation, in agreement with our previous studies [17, 18] and the known high stability of the Rho*- G_t complex [28].

Association and dissociation rates decreased even more, when the binding experiment was repeated on a preilluminated Rho-coated sensor surface (Figure 2c, black dotted line). The highly stable Rho*- G_t complex rapidly dissociated after injection of 100 μM GTP for 60 s. In general, the kinetics of Rho*- G_t dissociation were followed for up to 20 min (see an example in Figure 2d). The association kinetics was found to depend strongly on the number of Rho* (i.e. illumination time) as observed earlier [18]. This is apparent when comparing the sensorgrams obtained after 2 s and 4 s illumination (Figure 2c). However, the dissociation process also

varied with the illumination protocol. The initially fast phase in the dissociation process was due to the formation of dark Rho-G_t complexes that rapidly dissociated, and it significantly reduced when the illumination time was prolonged from 2 (gray solid line) to 4 s (black solid line).

By taking the concerted phenomena of G_t binding to either Rho or Rho* into account in the fitting procedure, the kinetic parameters for Rho*, reported in Table 1, were found to be independent on the illumination time. Interestingly, the association of G_t with dark Rho appeared to be approximately 1.6-fold faster compared with Rho*, whereas the dissociation of dark Rho-G_t complexes was found to be approximately 315-fold faster compared with the photoactivated complexes (Table 1). The rate constants used for comparison were the result of kinetic analyses performed on several replicates of the same binding experiment with different G_t concentrations and Rho immobilization techniques, as well as different amounts of immobilised Rho. The fitting procedure was performed according to the respective kinetic models (see material and methods) and gave satisfying results as shown in Figure 3.

Bleaching the pigment completely by applying 30-40 min of continuous illumination with the same light source allowed studying also the binding kinetics of G_t to opsin (Ops), the chromophore (ligand)-free form of Rho that is able to constitutively activate the phototransduction cascade with lower efficiency [29, 30]. The binding experiments with Ops showed a greater variability compared to the other receptor states, probably due to some intrinsic lower stability. Figure 4 shows a series of four replicates of the same 60 s injections of 0.33 μM G_t over Ops. Greater variability concerns mostly the maximum amplitude and to a lower extent the estimated rate constants. The kinetic analysis showed some interesting features (Table 1). While the association occurred approximately 1.8-fold faster for Ops compared to Rho*, the dissociation was approximately 3 times faster (but still 106-fold slower than in Rho, Table 1), hence resulting in a slightly lower affinity (1.8 ± 0.7 nM for Rho*, compared with 2.7 ± 1.5 nM for Ops). However, we point out the great instability observed in all the Ops experiments, which are reflected by the high relative error.

This on the first glance surprising and yet reproducible result appears to be at odds with the known catalytic potency of Ops toward G_t activation, which was shown to be 30-fold [31] to 10⁶-fold lower than that of Rho*[30]. However, SPR measurements allow only the evaluation of binding constants, not that of catalytic (enzymatic) rates. The data therefore reflect the difference between binding and activation of G_t to, respectively by Ops.

The interaction between Rho and G_t in the dark is a specific protein-protein interaction.

In order to probe whether the highly transient interaction detected between Rho and G_t in the dark could reflect unspecific binding of the G_t lipophilic anchors to potential lipids remained bound to solubilised receptors, we proceeded in two different ways. First, by employing liposomes containing the same lipid composition of bovine rod outer segment discs we immobilized on a sensor chip a phospholipid bilayer resembling the one in the discs, as done previously [23]. We then studied the direct interaction between G_t and the phospholipid bilayer in the same conditions as done in the presence of Rho (Figure 5). Injections of the same amounts of G_t led to different kinetics of interaction in the absence of Rho. The fast association was

followed by a significantly slower dissociation in which 25% of the G_t remained bound to the bilayer surface. In contrast, in all the experiments performed in the dark, a very fast and complete dissociation between Rho and G_t was observed (Figure 2 and Table 1). Second, we proved that after Rho immobilization, a set of 5 washing steps and further high flow stabilization leading to a $\sim 57,000$ -fold dilution in running buffer of the flow cell volume (FCV)(see Experimental) resulted in a highly efficient flow dialysis system. Indeed, the profiles shown in Figure 6 suggest that while in 5,000-fold FCV dilutions the amount of Rho was reduced by only 2% with respect to the initial immobilized level, phospholipids continuously dissociated from the flow cell upon further washing. The lipid concentration reached the detection limit already in this dilution frame, 11-fold lower than the one used in our binding experiments, (Figure 6), thus ensuring a substantial delipidation of Rho on the chip. These two lines of evidence rule out unspecific interactions involving lipids and point to a specific protein-protein interaction for Rho- G_t in the dark as detected by SPR, in line with previous observations [8-12].

Systems-level analysis of the phototransduction cascade in the presence of a dynamic scaffolding interaction between Rho and G_t .

The interaction between Rho and G_t in the dark was found to be highly transient and occurred through a fast association and an extremely fast dissociation (Table 1 and Figure 1b). How does this interaction process relate to the actual situation in photoreceptor cells? Two specific conditions of our on-chip experiments need particular attention: Rho is solubilised and delipidated and the interaction with G_t occurred in solution, in the volume of the sensor chip flow cell. Therefore, it is necessary to transform the volume-affinity constant measured here into one referred to surface-densities of Rho and G_t on rod discs [26]. By doing so (see Experimental), the seemingly low K_D^{dark} converts into $673 \mu\text{m}^{-2}$, leading to approximately 97% of G_t prebound with moderate affinity to Rho at equilibrium (taking approximately $3,000 \mu\text{m}^{-2}$ surface density of G_t). Nevertheless, this estimated value as well as the overall kinetic properties measured by SPR need to be considered in the context of the whole molecular machinery to assess the compatibility with the known dynamics of the phototransduction pathway.

The peculiar nature of Rho and specifically its ability to detect even single photons prevents any investigation at the cell level of the interactions between Rho and G_t in the dark by means of spectroscopic techniques such as FRET or BRET. However, the availability of a comprehensive dynamic model of phototransduction in rod cells, which was able to describe quantitatively the photoresponses starting from the underlying biochemistry [19] prompted us to test our findings in their general physiologic context. The mathematical model, validated over a broad set of experimental conditions involving different species and illumination ranges includes all the main components of the cascade (71 molecular species including proteins, ions and nucleotides) and the reactions between them that give rise to complex systems-properties such as light adaptation. Such detailed holistic description of the cascade activation/deactivation imposes strict constraints on the kinetic parameters in the model [19] and represents an ideal benchmark where to probe the physiological compatibility of our experimental finding at a whole network level.

In order to investigate Rho- G_t interaction in the dark, we hence included a further reaction (see Experimental) in the phototransduction network model described in [19] to describe a transient dark binding as observed by SPR. The relative kinetic constants instead of the absolute values of our kinetic analysis would be significant for this purpose, as they intrinsically account for the molecular determinants responsible for the different timing observed in the interaction between G_t and Rho or Rho*, independent of the environment. The new reaction thus followed the same relative kinetic relationships found in SPR experiments, that is $k_{dark}^{on} = 1.6 \times k_{light}^{on}$ and $k_{dark}^{off} = 315 \times k_{light}^{off}$ (Table 1).

Very interestingly, setting the above kinetic constraints for the precoupling reactions and simulating the system in the dark led to a very rapid equilibration, in which ~25% of the G_t were found to be dynamically precoupled to Rho in the dark, a value that significantly differs from the 97% estimated above, by neglecting the constraints imposed by the other components of the cascade and their dynamics. Hence, the numerical simulation at a systems-level suggests that anytime in the dark there is approximately a 38-fold excess of Rho uncoupled to G_t , compared to Rho- G_t able to capture a photon (or 97-98% of the overall available Rho). The binding/dissociation between the two proteins in the dark demonstrated experimentally is very fast and the simulations suggest that it leads to a rapid establishment of these equilibrium values. The continuous fast association and dissociation between Rho and to G_t in the absence of light stimulus is thus compatible with a “transient scaffolding” mechanism that is highly dynamic in nature.

Since a photon could hit either an isolated Rho molecule or one that is transiently coupled to G_t , the extended phototransduction network explicitly included the possibility for a flash light stimulus of fixed duration and intensity to be captured by: a) a Rho molecule uncoupled to G_t ; b) a precoupled Rho- G_t complex; c) both. The probability for the stimulus to be captured by one of the two forms (Rho or Rho- G_t) was set to depend on their relative abundance at the equilibrium in the dark (see Experimental). When the light stimulus was assumed to be proportionally absorbed by both forms, the shape and quantitative dynamics resulting from simulations were indistinguishable from those observed experimentally in rod cells (Figure 7a). Thus, the dynamic scaffolding hypothesis with the established kinetics is fully able to reproduce the electrophysiological data (see ref. [19] for direct comparison with experimental data) and is compatible with the kinetic constraints imposed by the whole network of interactions.

This systems-level analysis further allows simulating an ideal experiment to assess the possible effects on the timing of the overall cascade if only precoupled Rho- G_t complexes, and not free Rho, ideally captured the same light stimulus. Our simulations clearly show that the effect would be simply a reduction of the overall cascade efficiency, as the shape and the recovery of the photoresponses both appear normal (Figure 7a, red dashed lines). However, not a single biochemical event would be slowed-down, including the deactivation steps. Taken together, our simulations suggest that the stimulus might be captured by any of the Rho states, including the one precoupled with G_t , the only discriminator being the probability of such an event, which depends on the abundance of the preformed complexes relative to the available pigment and could be modified, for instance, in pathologic conditions.

Concluding remarks.

In conclusion, our findings are consistent with a dynamic scaffolding mechanism for Rho- G_t interaction, represented schematically in Figure 7b. Such dynamic mechanism does not require the presence of specific scaffolding proteins, which for instance is the case in *Drosophila* photoreceptor cells [16, 32]. The structural organization of the molecular machinery in vertebrate discs seems in fact sufficient for the purpose. The organization of Rho dimers in supramolecular architectures like the suggested paracrystalline rafts could facilitate the anisotropic diffusion of G_t in the restricted lipid areas among the receptor rafts despite the high concentration of Rho. This counterintuitive possibility, difficult to probe experimentally, is in fact supported by recent mesoscopic Monte Carlo simulations accounting for geometrical features of the diffusing proteins [33]. However, besides diffusing in the lipid milieu, G_t has a propensity toward protein-protein interactions with Rho in the dark. These two concerted and opposing diffusion/binding phenomena give rise to a dynamic “hopping” of G_t onto dark-adapted Rho, hence building up a convenient scaffold that maintains a dynamic equilibrium in which approximately 1 out of 4 G_t 's are actually bound to Rho at any time independent on the illumination state.

The mechanistic model proposed here seems to answer some of the questions raised on the coupling between G_t and Rho [34] and finally allows to consider the phototransduction cascade not as the exception among the many GPCR-mediated signalling pathways. In particular, the novel concept that interactions between the receptor and the G protein may occur any time independent of the stimulus also in the case of Rho and G_t is thought to be of relevance for retinal diseases involving photoreceptors. Future work is intended for exploring possible implications.

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Figure legends

Figure 1

SPR study of Rho-Rho interaction. In these two examples, approximately 10 ng Rho were immobilized on the sensor chip surface via amine coupling. After a short pulse of SDS to dissociate non-covalently bound oligomers, detergent-solubilized Rho dissolved in running buffer at different final concentrations was flowed over the chip for 3 min. Stable complexes formed at each concentration, following a slow but effective association. The bulk effect observed in both the association and dissociation phases and resulting in abrupt changes of SPR signal was due to the presence of CHAPS detergent in the injected solution, a more apparent phenomenon at higher Rho concentration. The data were fitted according to a simple 1:1 binding model, resulting in an average 17 nM affinity for Rho dimerization in the conditions tested here.

Figure 2

Transducin purification and SPR study of Rho-G_t binding. **a**, SDS-PAGE (12.5% polyacrylamide gel) after silver staining of G_t extracted from bovine retinae (see Experimental). The protein standard MW lane is shown together with the running lane showing the three G_t subunits. **b**, Example of SPR sensorgrams obtained in binding experiments. The concentration of G_t in the mobile phase was 0.33 μM and the immobilized Rho was 0.22 pmol mm⁻². The sensorgram relative to dark binding (black dotted line) is compared with that obtained after 4 s illumination of Rho (black solid line) and that obtained after 40 min bleaching (Ops, gray line). **c**, Prolonging the illumination time of the same Rho sample from 2 s (gray line) to 4 s (black solid line) leads to increased Rho*, that is higher response amplitude and relatively lower contribution of the fast dissociation phase due to the formation of dark Rho-G_t complexes. If the same illumination (4 s) and G_t binding experiment is repeated on a preilluminated Rho-coated sensor surface without prior GTP-induced dissociation of the Rho*-G_t complexes, a slower association and reduced response amplitude are observed (black dotted line). The notably slower dissociation phase is due to the highly reduced amount of free dark Rho. **d**, Rho*-G_t complexes (4 s illumination in this example) are very stable compared to the highly transient complexes in the dark. In this example, the dissociation of 0.33 μM G_t from 0.19 pmol mm⁻² immobilized Rho was followed for 20 min. In such time frame only 45 % of the bound complexes dissociated.

Figure 3

Examples of SPR kinetic analysis of dark and photoactivated Rho binding to G_t. **a**, Binding of 0.2 μM G_t to 0.19 pmol mm⁻² dark-adapted Rho. The dissociation curve (right) was fitted according to a single exponential, leading to $k_{dark}^{off} = 0.11 \text{ s}^{-1}$. The association (left) was described as a single pseudo-first order process (see Experimental), leading to $k_{dark}^{on} = 6.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. **b**, Binding of 0.33 μM G_t to the same Rho illuminated for 4 s before injection. The dissociation was fitted according to a double exponential, the first representing the fast dissociation of Rho-G_t

complexes. The fitting led to $k_{light}^{off} = 4.5 \times 10^{-4} \text{ s}^{-1}$. The association was modelled as a combination of two pseudo-first order terms (see Experimental), leading to $k_{light}^{on} = 3.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The fitting curves are shown in light gray.

Figure 4

Examples of four repeated 60 s injections of $0.33 \mu\text{M G}_t$ over a sensor chip coated with Ops after full bleaching of the formerly active visual pigment. At $t = 650 \text{ s}$ $100 \mu\text{M GTP}$ was injected leading to the complete dissociation of the Ops- G_t complex. The maximum amplitude significantly varies among the experiments, and the kinetic constants for each experiment were as follows (numbers refer to curves from top to down): $k_{1}^{on} = 3.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{1}^{off} = 5.6 \times 10^{-4} \text{ s}^{-1}$; $k_{2}^{on} = 4.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{2}^{off} = 5.5 \times 10^{-4} \text{ s}^{-1}$; $k_{3}^{on} = 4.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{3}^{off} = 5.2 \times 10^{-4} \text{ s}^{-1}$; $k_{4}^{on} = 6.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{4}^{off} = 2.2 \times 10^{-4} \text{ s}^{-1}$.

Figure 5

Example of interaction of $0.33 \mu\text{M G}_t$ with a bilayer with the same phospholipids composition as ROS discs (40% PE, 40% PC, 15% PS and 5% cholesterol; see Experimental). Flow and running buffers were the same as in all the experiments done in the presence of Rho. In every repeated experiment approximately 25% of G_t remained bound to the bilayer after injection.

Figure 6

Efficient flow dialysis of the flow-cell volume (FCV, $0.02 \mu\text{L}$), in which Rho was immobilized. In this example, 5.4 ng Rho were initially immobilized on the sensor chip flow cell via ConA. Fractions of $10 \mu\text{l}$ each of the solution flowing off the FCV were collected right after the immobilization was complete. The first point, corresponding to 500-fold FCV dilution is likely affected by the former washing of the recovery cup, hence resulting in more diluted sample, compared to the subsequent fractions. The washing profile of Rho bound to the chip (o) shows that only 2% of the initial amount is lost in the 5,000-fold overall dilutions. In contrast, the concentration of phospholipids in the fractions (\diamond) as compared to the initial amount (100%) continuously decreased upon subsequent washing steps, and was substantially negligible after 5,000-fold FCV dilution, hence ensuring a very effective delipidation of Rho on the sensor chip in the 57,000-fold FCV dilution achieved prior to any binding experiment.

Figure 7

Numerical simulation of rod photoresponses to flashes of increasing light intensities and dynamic scaffolding mechanism. **a**, Simulated photoresponses in an amphibian rod stimulated by 24 ms flashes of increasing intensities, ranging from 1.5 to 52,700 photoisomerizations. The resulting currents, expressing the difference between the dark current and the light-induced photocurrent, are shown for the realistic case in which the light stimulus is proportionally absorbed by both G_t -unbound Rho and preformed Rho- G_t complexes (black solid lines) and, ideally, by the preformed

complexes only (red dashed lines). In both cases, though with different efficiency, the photoresponse can reach saturation and for all the intensities it shows the typical deactivation kinetics. The details of the holistic biochemical model are explained in ref. [19]. **b**, Scheme of the dynamic scaffolding mechanism for Rho- G_t interaction in the dark. A patch of rod disc delimited by the membrane bilayer is represented and proteins other than Rho (green) and G_t (magenta) are omitted for clarity. At equilibrium, approximately 25% of G_t in the disc are dynamically bound to Rho, which is organized in supramolecular architectures such as the rafts of paracrystalline structure shown here, whose most common unit is the Rho dimer. The protein-protein scaffolding is highly dynamic, as a combined result of the diffusion of G_t in the lipid milieu by its farnesyl and acyl modifications and the high rate of dissociation/association from/to dark Rho. When the disc patch is exposed to light, a photon can either hit a preformed Rho- G_t complex or, with higher probability, a G_t -unbound Rho (bright green cylinders represent photoactivated receptors), in both cases triggering the activation of the phototransduction cascade

Tables

Table I: SPR kinetic parameters for G_t binding to dark and photoactivated Rho and Ops

	$k^{on} (M^{-1}s^{-1})^1$	$k^{off} (s^{-1})^1$	$K_D (nM)^1$
Rho ²	$(4.2 \pm 0.6) \times 10^5$	$(1.48 \pm 0.07) \times 10^{-1}$	360 ± 60
Rho* ³	$(2.7 \pm 0.5) \times 10^5$	$(4.7 \pm 1.5) \times 10^{-4}$	1.8 ± 0.7
Ops ⁴	$(5.0 \pm 0.7) \times 10^5$	$(1.4 \pm 0.6) \times 10^{-3}$	2.7 ± 1.5

¹ Data represent the mean (\pm s.e.m.) of replicate experiments, in which 0.33 μ M or 0.2 μ M G_t was injected for 60 s over a sensor chip and the dissociation followed for 120 s up to 20 min (see Experimental). Rho had an average density of 0.20 pmol mm^{-2} .
² Immobilization of Rho on the sensor chip was achieved via ConA or 1D4 antibody coupling. ² results from 19 replicate experiments. ³ results from 7 replicate experiments. ⁴ results from 9 replicate experiments













